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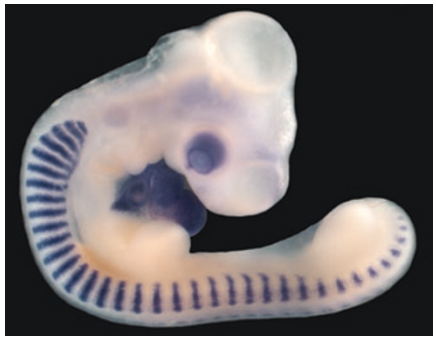
Size is not everything

They may be tiny, but microRNAs show strength in numbers and by exerting a surprising amount of influence over the expression of many genes. In the space of just a few years, the identification and analysis of microRNAs has become a boom industry, necessitating new tools and techniques suitable for such small targets. Michael Eisenstein reports.

It was surely a surprise when Victor Ambros and colleagues at Harvard University discovered that the *Caenorhabditis elegans* gene *lin-4*—previously known only as a negative regulator of other developmentally important genes—encoded a small untranslated RNA with apparent antisense properties[1]. All the same, such microRNAs scarcely seemed to be the herald of a biological revolution. "At the time, it seemed like an obscure way that the developmental timing of one cell in the vulva of a worm was being affected—and it's hard to think of anything more obscure than that," says Ronald Plasterk of the Hubrecht National Laboratory of Developmental Biology. "But now we think that a third of all human genes are regulated by microRNAs!"

The microRNA story has developed in parallel with the explosion of interest in RNA interference, and the realization that these two systems—one exogenous and one endogenous—are closely related from a mechanistic perspective. MicroRNA discovery has now become a boom industry, and public repositories of microRNA data have swelled rapidly within the space of only a few years (**Box 1**). At the same time, as Plasterk indicates, data increasingly suggest a broad and important role for these tiny transcripts in gene regulation, with microRNA genes and targets widely conserved across diverse species—even in viruses. "Any kind of process that you can look at, you can find a microRNA that's involved in some way or another," says Yale University's Frank Slack. "MicroRNAs are regulating metabolism, housekeeping genes, developmental genes, signaling genes—anything you can think of."

In light of the growing interest in this increasingly vibrant and exciting field of research, scientists and manufacturers are racing to develop quality-tested tools for discovery, isolation and characterization. "MicroRNA is still a small market," says Peter Roberts, product manager at Exiqon, "but it's growing rapidly, and there's a lot of interest."



In situ hybridization in a chick embryo, where LNA antisense probes have detected muscle-specific expression of *miR-1*. (Courtesy of D.K. Darnell and P.B. Antin, University of Arizona.)

Starting small

MicroRNAs are initially transcribed as relatively long primary microRNA transcripts (pri-miRNAs), which are processed into precursor molecules by the enzyme Drosha. These precursors are around 60 nucleotides in length and show partial self-complementarity that enables the formation of a stem-loop structure. The action of the Dicer enzyme on the precursor results in the generation of a 21-28-nt mature microRNA from a segment of the stem-loop. One strand of this is then loaded onto an RNA-induced silencing complex (RISC) in a fashion similar to small interfering RNAs. The initial identification of such small RNAs poses a fundamental challenge, and although computational prediction tools have been developed, such as MirScan and miRSeeker, the genomic characteristics of microRNA genes remain sufficiently poorly understood that experimental confirmation is essential.

Column-based size-separation methods, such as Qiagen's miRNeasy, Stratagene's miRACLE or the *mir*Vana miRNA Isolation kit from the Ambion reagents division of Applied Biosystems, are popular and affordable options for initial purification. These kits generally begin with a tissue-lysis step, followed by the use of a glass or silica matrix to separate smaller RNAs, typically 200 nt in length or less, from larger molecules. Ambion also offers the flashPAGE Fractionator for more precise isolation of a smaller size-range of molecules. This unit uses precast polyacrylamide gels for rapid fractionation of total RNA; within approximately 12 minutes, users can purify RNAs of 40 nt or less. Then, these can be concentrated via standard precipitation protocols or with the companion flashPAGE Clean-Up kit.

Work from the laboratories of David Bartel and Thomas Tuschl has yielded strategies for the cloning of Dicer-processed microRNAs from such size-selected populations, via stepwise ligation of specially designed adaptors followed by reverse transcription and PCR amplification[2,3]. The effectiveness of this and related approaches has been repeatedly demonstrated for the bulk identification of microRNAs, and Integrated DNA Technologies is among companies offering these linkers commercially. Effectiveness aside, however, some investigators describe these methods as time-consuming and challenging to optimize. "Every company that calls me to find out what they should be doing next, I've told them they should be making a cloning kit," says Slack, "because a lot more people would do it if it was easier."

At least one promising new alternative has emerged with the development of powerful new high-throughput sequencing methods, such as the platform developed by 454 Life Sciences, which make possible the rapid 'deep sequencing' of tremendous numbers of RNA sequences, providing bountiful leads for microRNA identification. "You can now isolate small RNA fractions at just the beginning stages of the cloning protocol and submit them to 454, and get back 200–300,000 reads, and be able to go to much greater depth and find rarer microRNAs," says David Bartel of the Massachusetts Institute of Technology.

Tracking tiny targets

For the first couple years of microRNA research, the old-fashioned northern blot was the method of choice for performing expression analysis, combining reasonable sensitivity with the ability to discriminate between mature microRNAs and unprocessed precursors—an important distinction, as some research suggests that microRNA processing

BOX 1 WHAT'S IN A NAME?

"In a way, we accidentally became the standard resource for microRNA information," confesses Sam Griffiths-Jones, project leader for the Wellcome Trust Sanger Institute's miRBase database. Their project was first conceived with a humbler goal: assigning names to validated microRNAs.

miRBase evolved as an offshoot of Rfam, a database of non–protein-coding RNA sequences. The issue of microRNA nomenclature had first emerged in 2003, when leading microRNA researchers proposed strict standards for microRNA annotation that took into account such factors as transcript structure, conservation and processing[10]. They further suggested that only transcripts matching a minimal, essential subset of these guidelines should be assigned an official microRNA name (for example, *miR-1*, *miR-2* and others). "It was post-genomic, in some sense—people have been through the pain of thinking about what protein genes should be called, and having to map those back through decades of literature," says Griffiths-Jones. "Because this was brand new, clearly it was the right time to impose some kind of system." It was this task that ultimately became the charge of the miRBase team.

However, miRBase has grown so rapidly and exercised such firm standards for registration—all microRNA submissions must be published in a peer-reviewed journal, or be a clearly conserved relative of a published microRNA to be named—that it has quickly become the undisputed, authoritative microRNA reference. The current database, version 8.2, contains detailed information on 4,039 pri-miRNAs and 3,834 mature microRNAs from organisms ranging from virus to human; a 20-fold increase over the first version, which launched in December 2003.

This is not to say that there have not been any snags. Many mature microRNAs differ by only a single nucleotide, and microRNA genes are often duplicated in higher organisms, making accurate naming of seemingly related microRNAs a potentially hairy process. "It's taken me a while to get to the point that there shouldn't be a line in the sand," says Griffiths-Jones. "It's a matter of judgment. And in fact, it's impossible, and indeed not sensible, to encode complex information about sequences in their names… if you are trying to infer complex biological information from the name, then you are probably going to be in trouble."

The miRBase team has recently bolstered their resource by partnering with Anton Enright's team at the Sanger to provide the TARGETS resource, which offers automated target prediction for all registered microRNAs. A second target database, TarBase, which only records experimentally verified microRNA targets—and verified nontargets—was recently launched by Artemis Hatzigeorgiou's group at the University of Pennsylvania[11], and discussions are underway regarding the future integration of these two databases into a combined resource. Both groups acknowledge that although a number of computational target prediction tools are presently available (see the Perspective in this issue, p. 881), further development will be essential. All the same, Hatzigeorgiou sees in these early efforts the seeds for effective future tools. "At this point, TarBase can be used as a good source for benchmarking computational prediction algorithms and, as it grows, for the application of machine learning approaches for target prediction," she says.

may be a key step in the regulation of their activity. Effective blotting of these small RNAs initially proved a challenge, but this changed with the development of effective membrane preparation techniques and kits for the production of short radiolabeled probes with high specific activity, such as Integrated DNA Technologies' StarFire labeling system. Ambion offers a different spin with the ribonuclease protection assay–based *mir*Vana miRNA Detection kit, in which probe and sample are hybridized in solution. Unhybridized RNA is eliminated by enzymatic digestion, allowing labeled target to be directly quantified on a gel, with no transfer step. Ambion claims this procedure offers a 100–500-fold increase in sensitivity over a standard northern blot.

Quantitative PCR (qPCR) is becoming popular for researchers examining rare microRNA species. "Real-time PCR provides a broader dynamic range, by several orders of magnitude, versus hybridization methods," says Criss Walworth, product line director for gene expression assays at Applied Biosystems. Applied Biosystems researchers recently described a new qPCR method, which uses a stem-loop–forming primer for the initial reverse-transcription step to achieve better specificity and sensitivity[4], and this is now the basis of the company's TaqMan MicroRNA Assays, which allow quantitation of as few as 200 copies of a given microRNA. Assays are available now for roughly 85% of the microRNAs listed in the Sanger miRBASE, and the collection is updated quarterly to include new content.

Exiqon also will be launching a new qPCR platform in the near future, incorporating their proprietary locked nucleic acid (LNA) chemistry. LNAs are modified RNA nucleotides in which the conformation of the sugar moiety has been altered so that LNA-hybridized nucleic acids assume an A-type rather than a B-type helix. "This is inherently more structurally stable, and that is expressed as an increase in melting temperature," explains Exiqon's Roberts. "At the practical level, you increase the melting temperature by between 2–8 °C." These properties make LNAs ideal for designing short qPCR probes, and have also made possible the analysis of microRNA expression via *in situ* hybridization. "LNA has been very enabling for this application," says Roberts. "We can say very confidently that before people were using LNAs, you just couldn't do it very well." Exiqon manufactures miRCURY LNA probes for virtually any miRNA sequence, and their effectiveness has been demonstrated in many studies, including recent work in zebrafish and mouse embryos from Plasterk's group[5]. "Within a couple of weeks, we had done all of the known and conserved microRNAs, and got really striking tissue specificity," Plasterk says. This has proven important, as evidence continues to accumulate that many microRNAs may be highly restricted in their expression. "We saw some that are expressed only in the rods of the eye or the cones or the lens or the eye muscle," says Plasterk, "so it's really very tissue-specific."

Micro chips

Microarrays may not provide the specificity of qPCR or *in situ* hybridization's capacity to precisely zoom in on sites of expression,

but the ongoing boom in microRNA gene discovery and increasing awareness of the combinatorial nature of microRNA-based gene regulation have fueled the hunger for higher-throughput systems for microRNA expression analysis. This is also a source of excitement for investigators working in diagnostics. "People have really taken notice of some of these papers saying that microRNAs give you a better readout of where a metastatic cell came from than other types of tests," says Slack, "and they've also taken notice of the fact that microRNAs can be good at making a prognosis for various diseases."

MicroRNA arrays are still a relatively new development, with many still being produced in-house by individual laboratories, but over the past two years a growing number of vendors has begun to offer array products. Although contemporary microRNA array experiments still face a variety of specific issues, such as the challenge of effectively labeling small oligonucleotide targets (see **Box 2**), they nevertheless offer researchers a quality-controlled—and routinely updated—alternative to the do-it-yourself chip.

Microarray specialist Combimatrix takes advantage of a fast and flexible *in situ* synthetic process to provide a broad range of microRNA arrays. "It's just-in-time manufacturing," explains Michael Tognotti, vice-president of sales and marketing. "If there's new content coming in or a new application, we can design it overnight, synthesize it overnight, and have it available for customers just as fast as one would order enzymes or custom oligos." Present offerings encompass the entirety of miRBase 8.2, with species-specific chips ranging from maize to human. The preparation process also makes the Combimatrix chips cost-effective. "Each microRNA array is only $99, and you get four uses out of it," says Tognotti, "so you can get an experiment done for around $25."

Ambion offers a variety of *mir*Vana microRNA Bioarrays based on GE Healthcare's CodeLink technology. These now cover the full range of human, mouse and rat microRNAs from miRBase version 8.0, as well as 152 'Ambi-miRs'—a set of proprietary human microRNAs. Oxford Gene Technology is also in the process of developing and testing designs and formats for its ink-jet–synthesized microRNA arrays as part of its participation in the Molecular Phenotyping to Accelerate Genomic Epidemiology (MolPAGE) consortium, an EU-funded initiative to identify biomarkers for human disease. Although these microRNA arrays have yet to be assembled into a formal collection of products, they are among the custom array options presently available to customers by request.

A primary challenge in working with microRNAs as targets for array analysis derives from their minimal length. "You only have around twenty-odd nucleotides to deal with; you can't target different parts like with an mRNA," says Roberts. "If you design capture probes based on DNA for all of those sequences, you'll end up with quite a wide range of melting temperatures based on complementarity." LNAs are one potential solution; melting temperature can be adjusted for each probe-target pair by modulating relative LNA content, and

The Trilogy 2020 instrument from U.S. Genomics uses fluorescence correlation spectroscopy for the sensitive quantification of microRNA targets. (Courtesy of U.S. Genomics.)

BOX 2 LABEL MAKERS

A fundamental challenge in performing microarray analysis of microRNAs is in the effective labeling of these molecules, owing both to their tiny size and to the absence of a poly(A) tail for use as a platform for generalized labeling. Several companies have responded by developing kits to enzymatically append artificial poly(A) tails onto microRNA targets. For example, Ambion's *mi*RVANA Labeling kit incorporates amine-modified nucleotides into such a tail, which are then treated with reactive fluorescent dye molecules. In contrast, Invitrogen's NCode Labeling System involves ligation of a poly(dT) 'capture' linker sequence onto this synthetic poly(A) tail, and this in turn is labeled with highly fluorescent DNA-based dendrimers, based on technology originally developed by Genisphere. Such kits are popular options, but some other manufacturers have pursued an alternative approach, using broadly applicable chemical labeling techniques that they believe may provide faster and more efficient alternatives.

One of these, the *Label* IT kit from Mirus, relies on alkylation chemistry for the covalent linkage of reactive Cy3 or Cy5 dye molecules to virtually any nucleic acid target. "It attaches to all of the different nucleotides," says James Hagstrom, vice president of scientific operations, "and it's controllable, depending on how much of the *Label* IT reagent you add." Mirus recently launched a *Label* IT kit for use with microRNAs, and according to the manufacturer, the kit offers a limit of detection as low as 0.08 femtomoles of target and is capable of overcoming some of the labeling biases that may exist with enzymatic kits. As an added benefit, *Label* IT is also suitable for use with plant microRNAs, which can not be tagged by standard enzymatic methods owing to endogenous 3′ methylation.

Kreatech Biotechnology offers an alternative chemical labeling strategy with their Universal Linkage System (ULS) platform. ULS uses coordination chemistry to couple platinum-conjugated fluorophores to guanine residues, forming a bond that, although not covalent, is nonetheless sufficiently strong and stable for microarray experiments. In addition to a rapid labeling procedure—15 to 30 minutes of incubation—Kreatech also boasts effective purification of excess dye molecules as a key benefit. "There is a cleanup column, the KREA*pure*, which is quite ideal for small RNAs because its purpose is to remove unreacted ULS reagents after the labeling reaction," explains product manager Anna McGeever. "It has no affinity for nucleic acids, so around 95% of the nucleic acids are recovered." Kreatech recently launched their *miR*acULS kit as well, which combines a column-based microRNA enrichment system with their labeling kit for a more integrated experimental workflow.

Although the choice of a labeling procedure may seem like a relatively minor step in the complex array experimental process, efficiency is a pressing issue, particularly for scarce targets like microRNAs, for which expression arrays offer a promising diagnostic tool. "It's important to have tools that are reliable," says Hagstrom, "and it's very important to get representative labeling so that you're not missing microRNAs in a detection experiment."

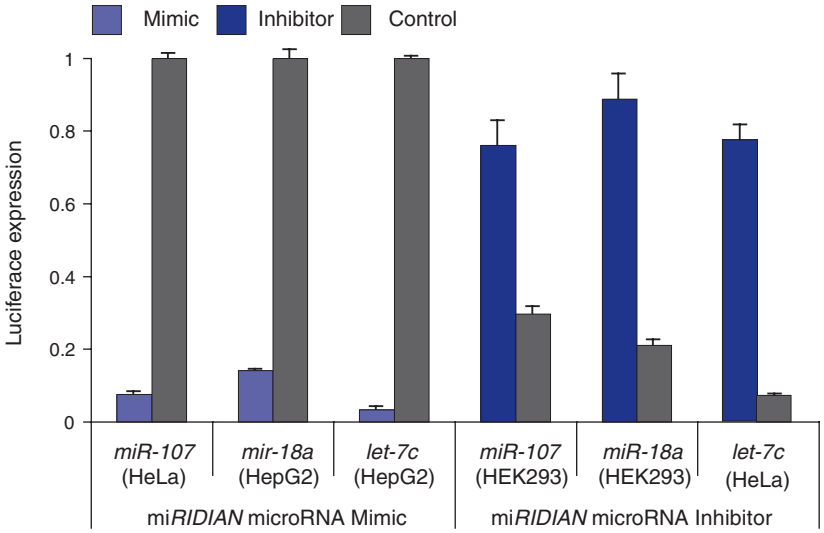
the high binding affinity allows the use of partial probes as short as 15 nt. Exiqon's miRCURY LNA arrays presently cover all of the human, the mouse and the rat sequences in miRBase 8.0, with each chip normalized to a hybridization temperature of 72 °C. miRCURY arrays can also be hybridized against total RNA, thereby requiring less sample and eliminating the need for a separate microRNA purification step.

Next-generation diagnostics

Microarrays hardly represent the final word on expression analysis, and a variety of other high-throughput tools with diagnostic promise have emerged as well. Zissimos Mourelatos and colleagues at the University of Pennsylvania developed their RNA-primed array-based Klenow enzyme (RAKE) assay with the aim of eliminating what they perceived as key weaknesses in the diagnostic accuracy of standard microarrays for microRNA analysis, such as potential bias introduced during microRNA amplification and labeling[6]. Each RAKE assay consists of an array of microRNA-complementary probes, where each antisense sequence is followed by several thymidine residues. The array is hybridized with total RNA, treated with exonuclease to eliminate unbound probes, and then incubated with Klenow enzyme and biotinylated ATP, such that only hybridized probes are labeled. RAKE makes large-scale quantitation more direct, combining the sensitivity of northern blots with the throughput of a microarray, and has proven a capable diagnostic tool for both freshly prepared and archival formalin-fixed paraffin-embedded samples.

An approach developed last year at the Broad Institute of the Massachusetts Institute of Technology and Harvard is a more radical departure from chip- and slide-based methods, relying instead on the flow-cytometric profiling of microRNA-bound polystyrene beads[7]. The researchers conjugated these beads to probes complementary to particular microRNAs, with each bead containing a specific combination of fluorescent dyes. They hybridized these conjugates against microRNA that had been amplified with biotinylated primers, and then stained with streptavidin-conjugated phycoerythrin. Flow-cytometric analysis of these samples revealed the microRNA being detected (via the combination of fluorophores contained in each bead) and the relative quantity of that microRNA (via the intensity of the phycoerythrin signal). This method proved to be a useful tool for identifying general trends in microRNA expression in cancerous tissues, as well as for the accurate classification of different types of tumors—more so than mRNA expression profiling—reinforcing the potential value of microRNA as a diagnostic tool.

U.S. Genomics also incorporates microfluidics and fluorescence detection into their *Direct* assays for microRNA quantitation[8]. Each microRNA is targeted by two LNA-containing probes, which label each end of the target with two different fluorophores. Samples are then analyzed with U.S. Genomics' Trilogy 2020 system; individual microRNAs are microfluidically directed through adjacent laser beams capable of exciting each fluorophore. Only molecules that produce consecutive signals for both fluorophores are considered 'hits', resulting in highly sensitive detection. The current batch of *Direct* assays have been



Examples of Dharmacon's mi*RIDIAN* Mimics and Inhibitors. The cell lines in each experimental group express a luciferase reporter under the regulation of the microRNA of interest. (Courtesy of Dharmacon.)

developed with an eye toward diagnostics, and U.S. Genomics recently entered into an arrangement with Rosetta Genomics for the development of assays for early lung-cancer diagnosis. Although Trilogy units can be purchased, U.S. Genomics has also launched a service business, which might prove more cost-effective for research and clinical customers. According to Duncan Whitney, vice president of research and development, Trilogy can detect even rare microRNAs in as little as 20 ng of total RNA; however, plans are in development for a far more sophisticated instrument. "In addition to providing a 100-fold multiplexing improvement, we hope to show that this can be done with tenfold less total RNA while providing a tenfold detection sensitivity improvement for each target," says Whitney.

Turn-ons and turn-offs

Of course, for many researchers, the strategies described above are merely appetizers for the experimental 'main course'—identifying microRNA function. Scientists hoping to characterize a new gene typically race to make a mouse knockout, but high levels of microRNA gene duplication in mammalian genomes pose an obstacle to this approach. "You can have four or five or six different versions of your microRNA, depending on the genome," explains Slack. "For example, it turns out that there are 12 human *let-7* genes." Fortunately, there are detours. For one, strong conservation across species means that phenotype analysis can sometimes be done by mutagenesis in simpler species like zebrafish or worm. Additionally, several companies have developed antisense inhibitors that allow functional characterization in model organisms or cell lines without the need for genomic tinkering.

It has been known for some time that 2′-*O*-methyl (2′-*O*Me) antisense oligonucleotides will bind with high affinity to RNA targets, and these molecules have proven useful for microRNA knockdown applications. More recently, Markus Stoffel and colleagues at Rockefeller University demonstrated that by conjugating a cholesterol moiety onto a 2′-*O*Me oligonucleotide, they could generate 'antagomirs' with improved pharmacokinetic properties that enable effective *in vivo* knockdown of microRNA targets[9], and this technology is now under development by Alnylam for therapeutic use in humans.

Morpholino oligos, which bind with high affinity and sterically interfere with microRNA action, also represent a potent option for *in vivo* blocking. Gene Tools, founded by morpholino coinventor James Summerton, now offers a catalog of special 'Multi-Blocking' morpholinos targeting every zebrafish microRNA in miRBase. "These are 31-base oligos targeting the pri-miRNA as well as the active miRNA sequence," explains Jon Moulton, head of Gene Tools' microRNA program. "Each overlaps the Drosha and Dicer cleavage sites and extends onto the loop of the pri-miRNA." This design strategy therefore targets the maturation and active stages of the microRNA life cycle, enhancing knockdown efficiency. Gene Tools also offers custom morpholinos, and will even assist in the design process. Likewise, Exiqon offers both catalog miRCURY LNA oligonucleotides for antisense knockdown—spanning the breadth of the Sanger database—and made-to-order sequences.

Dharmacon's mi*RIDIAN* products encompass tools for both knockdown and gain-of-function studies. mi*RIDIAN* Inhibitors comprise a roster of chemically-modified oligonucleotides targeting the full range of confirmed human, mouse and rat microRNAs with a level of efficiency that they claim surpasses standard 2′-*O*Me oligonucleotides. mi*RIDIAN* Mimics, in contrast, are dsRNA mimetics designed in a manner that ensures proper loading onto RISC and sequence targeting that simulates and augments that of the selected endogenous microRNA. "The mimetics employ advanced technologies that we developed to ensure that only the desired guide strand is used as a microRNA," explains William Marshall, group vice president of technology and business development for Fisher Biosciences. "The strand inactivation technology ensures that the Mimic performs similarly to the endogenous microRNA without any non-specific activity by the passenger strand." Dharmacon also offers ready-to-transfect, 96-well libraries of both Inhibitors and Mimics for use in high-throughput screens with cultured cell lines.

Even with such tools, some microRNAs stubbornly resist functional characterization, but the explanation for this may ultimately reside in the fundamental subtlety, and potential redundancy, of individual microRNA regulatory activity. "The factors of silencing are usually twofold or tenfold, but never a thousand-fold, it seems," says Plasterk. "And quite often, the messengers are targeted by different microRNAs at the same time—that makes it a wonderfully plastic system that can be used to fine-tune gene expression in a way that is probably the stuff of which evolution is made."

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Michael Eisenstein is technology editor for *Nature* and *Nature Methods*.

SUPPLIERS GUIDE: COMPANIES OFFERING MICRORNA RESEARCH PRODUCTS AND SERVICES

Company	Web address
Agilent	http://www.agilent.com
Alnylam	http://www.alnylam.com
Applied Biosystems (Ambion)	http://www.appliedbiosystems.com
AROS Applied Biotechnology	http://www.arosab.com
Asuragen	http://www.asuragen.com
BioDynamics Laboratory	http://www.biodynamics.co.jp/e
Cambridge BioScience	http://www.bioscience.co.uk
Cepheid (Actigenics)	http://www.cepheid.com
CombiMatrix	http://www.combimatrix.com
Dharmacon	http://www.dharmacon.com
Digital Genomics	http://www.digital-genomics.co.kr/eng
e-Oligos	http://www.e-oligos.com
Exiqon	http://www.exiqon.com
Full Moon Biosystems	http://www.fullmoonbio.com
Genaco	http://gene.genaco.com
Gene Link	http://www.genelink.com
Gene Tools, LLC	http://www.gene-tools.com
Genisphere	http://www.genisphere.com
GenoSensor Corp.	http://www.genosensorcorp.com
Hokkaido System Science	http://www.hssnet.co.jp
Integrated DNA Technologies	http://www.idtdna.com
Invitrogen	http://www.invitrogen.com
Isis Pharmaceuticals	http://www.isispharm.com
Kreatech Biotechnology	http://www.kreatech.com
LC Sciences	http://www.lcsciences.com
Millipore	http://www.millipore.com
mirMAX	http://cord.rutgers.edu/mirmax/index.html
Mirus Bio	http://www.mirusbio.com
Norgen Biotek	http://www.norgenbiotek.com
Open Biosystems	http://www.openbiosystems.com
Oxford Gene Technology	http://www.ogt.co.uk
PerkinElmer	http://las.perkinelmer.com
Qiagen	http://www.qiagen.com
Rosetta Genomics	http://www.rosettagenomics.com
Santaris Pharma	http://www.santaris.com
Sigma-Aldrich	http://www.sigmaaldrich.com
Stratagene	http://www.stratagene.com
System Biosciences	http://www.systembio.com
U.S. Genomics	http://www.usgenomics.com